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Peroxisome Proliferator-Activated Receptor-γ Is a Target of Nonsteroidal Anti-Inflammatory Drugs Mediating Cyclooxygenase-Independent Inhibition of Lung Cancer Cell Growth

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ABSTRACT

Nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit the growth of different cancer cell types, suggesting a broad role for their cyclooxygenase (COX) targets and eicosanoid products in tumor cell growth. Sulindac sulfide, a COX inhibitor, inhibited the growth of non–small-cell lung cancers (NSCLC) both in soft agar and as xenografts in nude mice. Importantly, the concentration of sulindac sulfide required to inhibit NSCLC cell growth greatly exceeded the concentration required to inhibit prostaglandin (PG) E_2 synthesis in NSCLC cells, suggesting that NSAID inhibition of cell growth is mediated by additional targets distinct from COX. Both sulindac sulfide and ciglitazone, a defined peroxisome proliferator-activated receptor-γ (PPARγ) agonist, stimulated a promoter construct containing a PPAR response element linked to luciferase and potently inhibited NSCLC cell growth at similar concentrations, indicating a role for PPARγ as a target of NSAID action in these cells. Overexpression of PPARγ in NSCLC cells strongly inhibited the transformed growth properties of the cells, providing a molecular confirmation of the results obtained with the PPARγ agonists. Increased expression of PPARγ, as well as ciglitazone and sulindac sulfide induced expression of E-cadherin, which has been linked to increased differentiation of NSCLC. Despite the fact that SCLC cell lines expressed little or no cytosolic phospholipase A_2, COX-1, or COX-2, sulindac sulfide and PPARγ agonists also inhibited the transformed growth of these lung cancer cells. We propose that PPARγ serves as a target for NSAIDs that accounts for COX-independent inhibition of lung cancer cell growth.

Nonsteroidal anti-inflammatory drugs (NSAIDs) are a class of compounds that block eicosanoid production through the inhibition of cyclooxygenase (COX) activity (Smith et al., 1994). In addition to their general use as inhibitors of inflammation, pain, and fever, NSAIDs have an emerging utility as chemotherapeutics for the prevention and treatment of human cancer (Marnett, 1992; Duperron and Castonguay, 1997). The observed chemoprevention of colon cancer by the NSAID sulindac (Rao et al., 1995) and epidemiological studies indicating that NSAIDs decrease the risk for developing lung cancer (Schreinemachers and Everson, 1994) are consistent with an emerging role for eicosanoid biosynthetic pathways in human cancer development.

A large number of studies have now demonstrated that NSAIDs may exert some of their cellular actions through COX-independent mechanisms (reviewed in Tegeder et al., 2001). Among these potential targets of NSAIDs is the peroxisome proliferator-activated receptor (PPAR) family of nuclear receptors that function as ligand-dependent transcription factors (Spiegelman, 1997). Three isoforms have been described, PPARα, -γ, and -δ, all of which bind to specific DNA sequences as heterodimers with the retinoic acid X-receptors (DiRenzo et al., 1997). PPARγ has been shown to be activated by the synthetic antidiabetic thiazolidinediones, such as ciglitazone and troglitazone (Lehmann et al., 1995), as well as by prostaglandin D and J derivatives, which may function as endogenous activators (Forman et al., 1995). Whereas the function of PPARγ in the setting of human cancer is controversial, recent findings indicate that loss of PPARγ expression is associated with colon tumorigenesis, and activation of PPARγ leads to inhibition of anchorage-independent growth of colon cancer cell lines (Brockman et

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ABBREVIATIONS: NSAID, nonsteroidal anti-inflammatory drug; COX, cyclooxygenase; PPAR, peroxisome proliferator-activated receptor; SCLC, small-cell lung cancer; NSCLC, non–small-cell lung cancer; $cPLA_2$, cytosolic phospholipase A_2; PPAR-RE, peroxisome proliferator-activated receptor–response element; TTBS, Tris-buffered saline-Tween 20; NF-κB, nuclear factor κB; PG, prostaglandin; APC, adenomatous polyposis coli; β-gal, β-galactosidase.

al., 1998), suggesting that this gene may function as a tumor suppressor.

Lung cancer is a heterogeneous disease that is generally categorized into small-cell lung cancer (SCLC) and non–small-cell lung cancer (NSCLC). As a group, the NSCLCs constitute the bulk of lung cancers and are subdivided into squamous, adenocarcinoma, and large-cell carcinoma phenotypes. Gain-of-function mutations in K-Ras are observed in approximately 30% of adenocarcinomas and just under 10% of other NSCLC types (Giaccone, 1996). These mutations seem to be virtually absent in SCLC (Mitsudomi et al., 1991). We and others have previously reported that a subset of NSCLC cell lines expressing oncogenic forms of Ras exhibit high levels of prostaglandin production, whereas SCLC cell lines produce little or no prostaglandins (Heasley et al., 1997). High levels of prostaglandin production by NSCLC cells are correlated with increased expression of both cytosolic phospholipase A_2 ($cPLA_2$) and COX-2 (Heasley et al., 1997). Moreover, expression of gain-of-function Ras was both necessary and sufficient to mediate increased transcription of these enzymes (Van Putten et al., 2001).

Based on the restricted expression of $cPLA_2$ and COX-2 and synthesis of prostaglandins by lung cancer cells noted in our studies and in the literature, a selective action of NSAIDs on various lung cancer cells would be predicted. In fact, preliminary studies in our laboratory revealed a widespread inhibitory action of NSAIDs on NSCLC and SCLC cell lines. In this study, we have examined the role of PPARγ as a potential target of NSAIDs mediating growth inhibition of diverse lung cancer cells. In light of multiple potential effects of both NSAIDs and PPAR activators, we employed both pharmacological and molecular approaches to assess the role of this pathway as a target of NSAIDs mediating the inhibition of transformed growth of NSCLC and SCLC cells.

Materials and Methods

Materials. Antibodies to PPARγ, $cPLA_2$, COX-1, COX-2, and E-cadherin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Sulindac sulfide, NS-398, ciglitazone, and WY 14,463 were purchased from Biomol (Plymouth Meeting, PA). The PPARγ-Gal4 expression plasmid was a generous gift of Dr. Jeffrey Flier (Beth Israel Hospital, Boston, MA). Expression plasmids encoding PPARγ and constructs encoding a consensus PPAR-response element ligated to a luciferase reporter (PPAR-RE) were the gift of Carl Clay (Wake Forest University Baptist Medical Center, Winston-Salem, NC.

Cell Culture and Transfection. Non–small-cell lung cancer cell lines (H2122, A549, H460) and small-cell lung cancer cell lines (H345, SHP-77) were obtained from the University of Colorado Health Sciences Center Cancer Center Tissue Culture Core. H2122, A549, H460, and SHP-77 cells were maintained in RPMI containing 10% fetal bovine serum and H345 cells were grown in HITES medium (RPMI medium containing 10 nM hydrocortisone, 5 μg/ml insulin, 10 μg/ml transferrin, 10 nM 17β-estradiol, 30 nM sodium selenite, and 0.1% bovine serum albumin). Cells were transfected by electroporation as described previously (Heasley et al., 1997). Two million cells were electroporated in 0.4-cm electroporation cuvettes (Bio-Rad, Hercules, CA) using a geneZAPPER (IBI, Madison, WI). After electroporation, cells were incubated in standard media for 48 h. Cells were then harvested and firefly luciferase and β-galactosidase activity determined as described previously (Heasley et al., 1997). Results are expressed as luciferase units normalized to milliunits of β-galactosidase. For stable transfections, the PPARγ1 cDNA (Gurnell et al., 2000) was inserted into the $pLNCX_2$ retroviral expression vector (BD Biosciences Clontech, Palo Alto, CA) and transfected into 293T cells along with vectors encoding gag, pol, and env proteins to make recombinant virus, as described previously (Van Putten et al., 2001). Medium from the 293T cells was used to transfect the ecotropic retroviral-producing GP+E-86 cell line, then medium from the infected GP+E-86 cells was used to transfect the amphotropic retroviral-producing packaging cell line, PA317. Medium from the LNCX2-PPARγ PA317 packaging cell line was used to stably transfect H2122 cell lines, as described above. Polybrene (8 μg/ml) was added to the retrovirus-containing medium collected from the packaging cells and filtered before two sequential 24-h incubations with subconfluent layers of cells. The infected cells were replated, selected for G418 resistance, and expanded. Clones were screened for expression of PPARγ by immunoblotting with a specific anti-PPARγ antibody. Control cell lines ($pLNCX_2$) were selected by infecting cells with a virus lacking a cDNA insert.

Growth Assay and Tumor Cell Growth in Athymic Mice. For determination of anchorage-independent growth, single-cell suspensions of the indicated NSCLC or SCLC lines were prepared and aliquots containing 10,000 cells were suspended in 1.5 ml of RPMI 1640 medium containing 10% fetal bovine serum and 0.3% Nobel agar and layered over a base prepared in 35-mm dishes of RPMI 1640 medium, 10% fetal bovine serum, and 0.5% agarose supplemented with the various inhibitors at twice the indicated concentration. For H345 cells, HITES medium (RPMI 1640 medium with the following additives per liter: 0.005 mg/ml insulin, 0.01 mg/ml transferrin, 30 nM sodium selenite, 10 nM hydrocortisone, 10 nM β-estradiol, 10 mM HEPES, and 2 mM L-glutamine) was used. The dishes were incubated for 3 to 4 weeks at 37°C in a humidified CO_2 incubator. Live colonies were stained for 5 to 20 h at 37°C with nitro blue tetrazolium chloride (1 mg/ml), visualized under a microscope, and counted. For determination of growth under standard conditions, cells were plated in 96-well plates. After 24 h, various concentrations of inhibitors were added. Cells were assayed for live cells 72 h later by the CellTiter 96 AQueous One Solution Cell Proliferation Assay ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay; Promega, Madison, WI). Results are given as percentage of live cells. For studies of tumor growth in vivo, athymic mice were inoculated subcutaneously in the flanks with the indicated tumor cells (10^7 cells/flank). Seven days after inoculation, mice were treated daily with sulindac sulfide (5 mg/kg) or vehicle administered intraperitoneally. Seven animals were used per treatment and tumor volumes were measured every 3 days.

Immunoblot Analyses. Cells were collected in phosphate-buffered saline and, after centrifugation (5 min, 1,000*g*), were lysed in mitogen-activated protein kinase lysis buffer (Heasley et al., 1996). Nuclei and cell debris were removed by microcentrifugation (5 min, 10,000*g*) and portions containing 100 to 200 μg of protein were mixed with SDS sample buffer and submitted to SDS-PAGE on 7.5%

acrylamide gels. The resolved polypeptides were transferred electrophoretically to nitrocellulose (MSI, Westboro, MA) and the filters were blocked extensively in Tris-buffered saline containing 0.1% Tween 20 (TTBS) and 3% nonfat dry milk. After an incubation (16–24 h) with the indicated antibodies in TTBS/3% milk, the filters were washed with four changes of TTBS and bound antibodies were visualized with horseradish peroxidase-coupled secondary reagents and enhanced chemiluminescence according to the manufacturer's specifications.

Results

We have previously reported that a subset of NSCLC cell lines expressing gain-of-function *K-ras* mutations express high levels of $cPLA_2$ and COX-2, leading to marked PGE_2 synthesis (Heasley et al., 1997). Anchorage-independent growth of these NSCLC cell lines, as assessed by colony formation in soft agar, was inhibited by inclusion of the NSAIDs sulindac sulfide or indomethacin (Table 1), suggesting that eicosanoid-generating pathways contribute to the transformed growth of NSCLC cells. To confirm the ability of these agents to block transformed growth of NSCLC, sulindac sulfide was tested for its ability to inhibit tumor growth in a xenograft model. This model is a more stringent criterion of transformed growth than growth in soft agar. As shown in Fig. 1, sulindac sulfide significantly reduced the growth of tumors arising from inoculation of the NSCLC line A549 in nude mice (Fig. 1). Thus, NSAIDs such as sulindac sulfide are effective inhibitors of transformed cell growth of NSCLC cells.

It is notable that the concentrations of NSAIDs required to inhibit soft agar growth shown in Table 1 are significantly higher than those required to inhibit prostaglandin production in these cells. We determined by radioimmunoassay that sulindac inhibited PGE_2 production in H2122 and A549 cells with an IC_{50} ~1 μM (data not shown), a concentration 20- to 100-fold lower than the concentrations required to inhibit anchorage-independent growth. In this regard, higher concentrations of NSAIDs have been reported to affect a number of other targets distinct from COX isoforms (for review, see Tegeder et al., 2001). We therefore undertook an examination of other potential NSAIDs effectors. Recent reports have demonstrated an ability of NSAIDs to inhibit IκB-kinase (Plummer et al., 1999), thereby resulting in an inhibition of NF-κB activity in cells. To test this possibility, NSCLC cells were transfected with a construct encoding three tandemized NF-κB consensus elements ligated into a luciferase reporter and stimulated with NSAIDs. Sulindac sulfide failed to significantly alter NF-κB activity (normalized luciferase activity: control, 13798; Sulindac sulfide, 12733). Similarly, this agent did not affect basal activities of the extracellular signal-regulated kinase or the c-Jun NH_2-terminal kinase family of mitogen-activated protein kinases (data not shown).

Specific members of the peroxisome proliferator-activated receptor (PPAR) family of nuclear receptors have been previously identified as targets of NSAIDs (Lehmann et al., 1997). Activation of these receptors is associated with growth arrest and differentiation of adipocytes (Spiegelman, 1997). Furthermore, both NSCLC and SCLC cell lines have been shown to express PPARγ (Tsubouchi et al., 2000). To directly test the ability of NSAIDs to activate PPARγ, NSCLC cell lines were transiently transfected with a construct encoding tandemized PPAR-response elements ligated to a promoterless luciferase construct (PPAR-RE), and exposed to either ciglitazone, a well-characterized PPARγ-activator, or sulindac sulfide. Both ciglitazone and sulindac sulfide significantly increased promoter activity in A549 and H2122 cells (Fig. 2A), consistent with previous reports documenting activation of PPARγ by NSAIDs (Lehmann et al., 1997). The ability of NSAIDs to function as PPARγ activators was confirmed by transfecting A549 cells with a construct encoding the activation domain of PPARγ fused to the DNA binding domain of the yeast transcription factor Gal4 (PPARγ-Gal4), along with a reporter plasmid containing five Gal4 binding sites upstream from a promoterless luciferase construct (UAS-luc). Cells were then exposed to ciglitazone or sulindac sulfide and luciferase activity was measured 24 h later. Both of these compounds significantly increased luciferase activity in cells cotransfected with PPARγ-Gal4 and UAS-luc (Fig. 2B). Thus, the findings in Fig. 2, A and B, demonstrate functional expression of PPARγ in NSCLC cells and that PPARγ can be activated by NSAIDs.

TABLE 1

Inhibition of soft agar colony formation by lung cancer cells

Replicate plates of the respective NSCLC or SCLC cell lines were plated in soft agar at 10,000 cells per well in the presence of various concentrations of sulindac sulfide or indomethacin. Colonies were counted after 3 weeks and the IC_{50} values were calculated.

	IC_{50}	
Cell Line	Sulindac Sulfide	Indomethacin
	μM	
NSCLC		
H2122	20	27
A549	80	>100
H460	29	40
SCLC		
SHP-77	8	5
H345	20	18

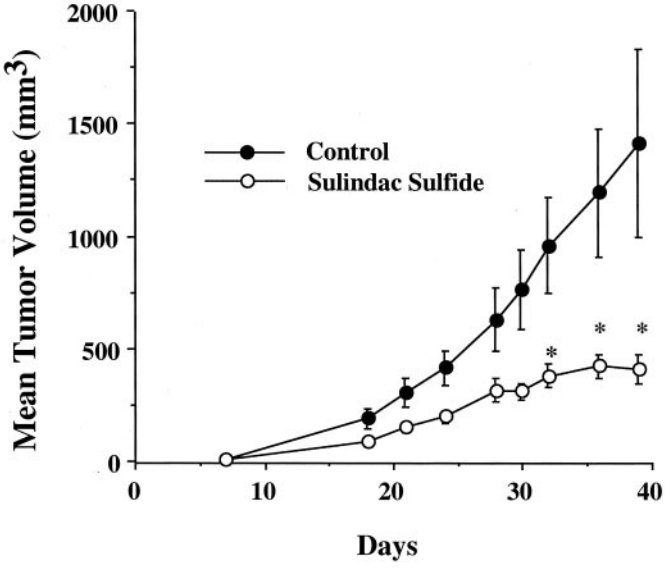

Fig. 1. Effect of NSAIDs on tumor growth in a xenograft model. Athymic mice were inoculated subcutaneously with 1×10^7 A549 cells. Seven days after inoculation, mice were treated daily with sulindac sulfide (5 mg/kg), or vehicle administered intraperitoneally. Tumor volume was measured every 3 days using calipers. Results shown are the mean ± S.E.M., where each group contained seven animals. *, $p < 0.05$ versus control.

The expression of PPARγ in NSCLC coupled with the ability of NSAIDs to increase the transactivation potential of PPARγ suggests that the effect of these agents on transformed growth of NSCLC cells may be mediated at least in part through activation of PPARγ. We therefore examined the effects of PPAR activators on anchorage-independent growth of NSCLC cell lines. Three defined PPARγ activators, ciglitazone, PGA_1, and 15-deoxy-Δ12,14-PGJ_2 (Forman et al., 1996) potently inhibited anchorage-independent growth of NSCLC cells at concentrations that are consistent with their EC_{50} values as PPARγ agonists (Chang and Szabo, 2000) (Fig. 3). By contrast, WY 14,463, a PPARα-specific agonist, showed little or no ability to inhibit colony growth (Fig. 3).

To more conclusively implicate PPARγ as the NSAID target mediating inhibition of NSCLC transformed growth, we established NSCLC lines that stably overexpressed a PPARγ cDNA by retroviral-mediated gene transfer (see *Materials and Methods*). Transfected NSCLC clones selected for resistance to G-418 were immunoblotted for PPARγ to identify those clones expressing the exogenous PPARγ polypeptide (data not shown). Of 24 clones examined, the three showing the highest level of PPARγ expression were selected for further study. Control cell lines (Neo) were transfected with construct lacking an insert. Functional overexpression of PPARγ in stable H2122-PPARγ clones was determined by transfecting cells with the PPAR-RE reporter and treating cells with either ciglitazone or sulindac sulfide. As shown in Table 2, overexpression of PPARγ in three representative stable H2122-PPARγ cell lines resulted in a marked increase in basal and stimulated PPAR-RE promoter activity compared with cells transfected with empty vector (Neo) or parental untransfected H2122 cells (not shown), consistent with the functional over-expression of PPARγ in H2122 cells. We then examined whether over-expression of PPARγ in H2122 cells influenced their transformed growth properties as well as their sensitivity to NSAIDs. As shown in Fig. 4, two independent clones overexpressing PPARγ failed to form colonies in soft agar. Another clone formed significantly fewer colonies than cells transfected with empty vector (Neo) or untransfected H2122 cells, and growth in soft agar was inhibited at significantly lower concentrations of sulindac sulfide. The PPARγ transfectants grew with similar doubling times as the Neo control cells on plastic tissue culture dishes in regular growth medium. Thus, this result demonstrates that increasing PPARγ activity in NSCLC cells by virtue of

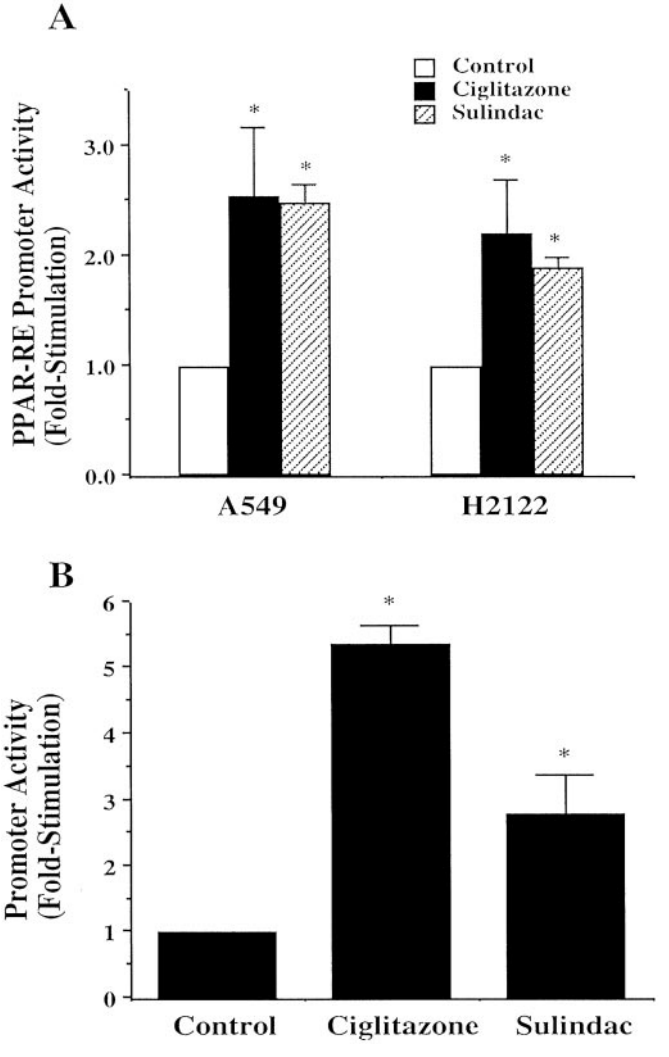

Fig. 2. Activation of PPARγ in lung cancer cells. A, the indicated NSCLC lines (A549 and H2122) were transiently transfected with the PPAR-RE, along with cytomegalovirus–β-gal to normalize for transfection efficiency. After an overnight incubation, cells were stimulated for 24 h with either 50 μM ciglitazone or 100 μM sulindac sulfide. Extracts were prepared and promoter activity determined as luciferase units normalized to β-gal. Results represent the mean of three independent experiments with the S.E.M. indicated. B, A549 cells were transfected with 10 ng of a plasmid encoding the activation domain of PPARγ fused to the DNA binding domain of Gal4 (PPARγ-Gal4) as well as 2 μg of a plasmid encoding five copies of the Gal4 binding site upstream from a luciferase reporter (UAS-luc) and 2 μg of CMV-βGal. After incubation overnight in normal media, cells were treated for 24 h with 40 μM ciglitazone, 50 μM sulindac sulfide, or vehicle (control). Lysates were prepared and assayed for luciferase and β-gal activity and promoter activity calculated as luciferase units/β-gal. Results are reported as -fold induction compared with vehicle-treated cells and represent the mean ± S.E.M. of four separate transfections with duplicate dishes. *, $P < 0.05$ versus control.

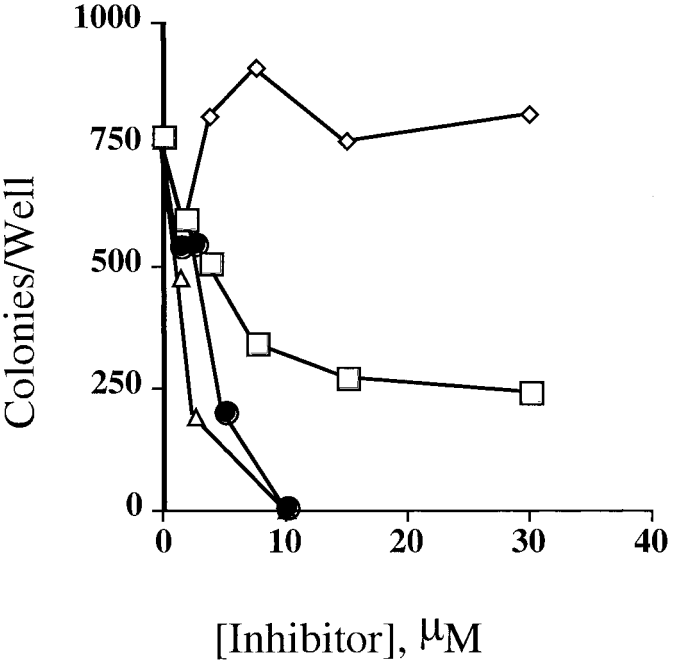

Fig. 3. Effect of PPAR activators on soft agar colony formation. H2122 cells were grown in soft agar in the presence of the indicated concentrations of ciglitazone PGA_1, PGJ_2, or WY 14,463. Colony formation was determined after 3 to 4 weeks as described under *Materials and Methods* section. □, ciglitazone; ◇, WY 14,1463; ●, PGA_1; △, PGJ_2.

TABLE 2

PPAR-RE promoter activity in H2122 stable transfectants

H2122 cells were stably transfected with retroviruses encoding full-length PPARγ and individual clones were selected for resistance to G-418. Control cells (Neo) were transfected with a retroviral vector lacking an insert. Three clones overexpressing PPARγ and one Neo clone were transiently transfected with the PPAR-RE promoter construct as described in Fig. 2. Cells were stimulated for 24 h with either 10 μ M ciglitazone or 25 μ M sulindac sulfide, and promoter activity normalized to β -gal was determined. Results represent the mean of three independent experiments.

	Luciferase Units/β-gal		
Cell Line	Basal	Ciglitazone	Sulindac Sulfide
Neo	6,617 ± 1,493	11,246 ± 3,256	10,242 ± 1,591
PPARγ-16	20,236 ± 1,056	99,747 ± 7,400	53,879 ± 4,097
PPARγ-18	58,805 ± 4,778	258,823 ± 3,4120	136,556 ± 3,516
PPARγ-3	54,517 ± 3,608	91484 ± 31,348	99,902 ± 19,766

overexpression dramatically reverses the transformed phenotype of these cells.

These experiments highlight the ability of NSAIDs to inhibit cancer cell growth through targets such as PPARγ that are distinct from COX inhibition. We next sought to examine the effect of NSAIDs on SCLC transformed growth. Initial studies were performed to characterize the status of eicosanoid-synthesizing pathways in SCLC lines. Comparison of cellular levels of $cPLA_2$, COX-1, and COX-2 in two SCLC lines (H345 and SHP-77) revealed low or undetectable levels of $cPLA_2$, COX-2, and COX-1 in the SCLC lines compared with the NSCLC lines H2122 and A549 (Fig. 5). Moreover, these cells produced no detectable prostanoids (data not shown), a finding consistent with previous reports noting the absence of prostaglandin synthesis in SCLC cells relative to NSCLC cells (Hubbard et al., 1989). Based on the lack of prostanoid synthetic pathways in SCLC, a high degree of sensitivity to inhibitors of prostaglandin synthesis was not predicted. In fact, diverse NSAIDs potently inhibited the anchorage-independent growth of SCLC lines SHP-77 and H345 (Fig. 6). Sulindac sulfide inhibited soft agar colony formation with an IC_{50} value that was lower than the IC_{50} value observed in NSCLC cell lines (Table 1). Because PPARγ is also expressed in SCLC (Tsubouchi et al., 2000), we tested the effect of defined PPARγ activators on transformed growth of these cells. As was observed in NSCLC, ciglitazone, PGA_1, and 15-deoxy-Δ12,14-PGJ_2 potently inhibited soft agar colony formation of SHP-77 and H345 cells (Fig. 6), whereas the PPARα activator WY 14,463 had no effect. Thus, the ability of NSAIDs to activate PPARγ probably accounts for the action of this class of compounds on tumor cells in which eicosanoid biosynthesis is not apparent.

If sulindac sulfide and ciglitazone are acting through overlapping pathways involving PPARγ, it would be anticipated that a number of genes would be induced by both classes of agents. We have undertaken a preliminary screen to identify such genes. We observed that exposure of H2122 cells to either sulindac sulfide or ciglitazone for 48 h resulted in a marked induction of E-cadherin (Fig. 7B). Increased expression of E-cadherin in response to PPARγ activators has been

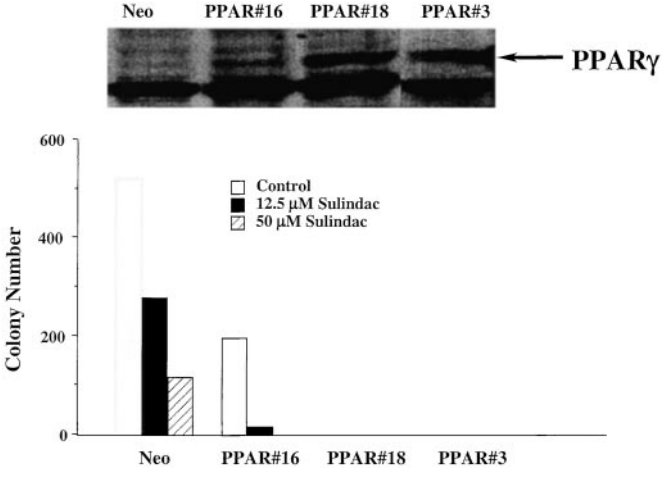

Fig. 4. NSCLC overexpressing PPARγ show markedly reduced growth in soft agar. H2122 cells transfected with empty vector, or three independent clones (PPAR-16, -18, and -3) overexpressing PPARγ were grown in soft agar in the presence of the indicated concentrations of sulindac sulfide. After 3 weeks, colonies were visualized and counted as described under *Materials and Methods*. Results represent the mean of duplicate experiments with two wells per condition in each experiment. Clones 18 and 3 failed to form any colonies containing viable cells. Inset, extracts were prepared from the indicated cell lines and immunoblotted with anti-PPARγ antibody. The arrow indicates the migration of recombinant PPARγ.

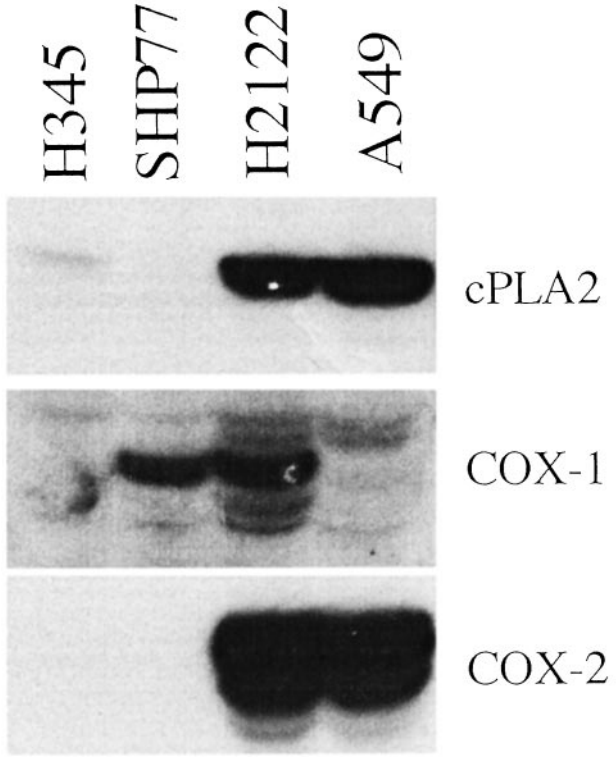

Fig. 5. Immunoblots of $cPLA_2$, COX-1, and COX-2 in NSCLC and SCLC cell lines. Extracts were prepared from the SCLC cell lines H345 and SHP-77 as well as the NSCLC lines H2122 and A549 as described under *Materials and Methods*. The proteins were resolved by SDS-polyacrylamide gel electrophoresis and immunoblotted with antibodies directed against $cPLA_2$, COX-1, and COX-2. The bound antibodies were visualized with horseradish peroxidase-coupled secondary antibodies and developed with enhanced chemiluminescence.

reported in pancreatic cancer cells and has been hypothesized to be involved in differentiation of these cells associated with decreased tumorigenic potential (Ohta et al., 2002). Increased expression of E-cadherin was also observed in H2122 cells stably overexpressing PPARγ (Fig. 7A).

Discussion

A key role for eicosanoid biosynthetic pathways in human cancer development is supported by numerous reports in the literature (Dannenberg and Zakim, 1999; Marks et al., 1999). Clearly, induction of COX-2 and $cPLA_2$ is observed in colonic polyps and carcinomas (Kargman et al., 1995). Moreover, chronic NSAID intake reduces colon cancer incidence in animal models and humans. Colon cancer incidence in the setting of adenomatous polyposis coli (APC) deficiency is markedly reduced in COX-2 deficient (Oshima et al., 1996), and $cPLA_2$-deficient mice (Takaku et al., 2000), and overexpression of COX-2 has been shown to be sufficient for induction of mammary tumors (Liu et al., 2001). We have previously reported enhanced PGE_2 production in NSCLC cell lines that correlated with the expression of oncogenic Ras mutations (Heasley et al., 1997). This was mediated through increased expression of $cPLA_2$ and COX-2 proteins. The induction of COX-2 has also been verified in primary human lung cancer specimens (Hida et al., 1998). Thus, $cPLA_2$ and COX-2 are induced in diverse cancer cells with apparently critical roles in the transformed growth properties of the tumor cells. Whereas the mechanism whereby enhanced prostaglandin production contributes to transformed growth, recent evidence has been presented suggesting transactivation of EGF receptors (Pai et al., 2002).

If the effects of NSAIDs on cancer cell growth are mediated solely through inhibition of COX, than exogenous addition of prostaglandins would be predicted to overcome the growth inhibition. These experiments have not been reported to date, suggesting that at least some of the growth effects of NSAIDs are mediated through alternative targets. The observation that significantly higher concentrations of NSAIDs are required to inhibit growth of NSCLC cells, coupled with the ability of these agents to inhibit growth of tumor cells such as SCLC cells, which generate no detectable prostaglandins further argues that this class of drugs has targets other than COX-1 or COX-2. The study of Kliewer et al. (1995) provides strong evidence for PPARγ as a target of NSAIDs. Our finding that a PPARγ agonist (ciglitazone), but not a PPARα agonist inhibits lung cancer cell growth provides additional support for the view of PPARγ as a functional target for diverse NSAIDs in inhibiting the growth of lung cancer cells. Interpretation of many of these experiments is complicated by the possibility that drugs that activate PPARγ may also act on additional targets. To address this issue, we have also employed a molecular strategy in this study by overexpressing PPARγ in NSCLC. Multiple clones of H2122 cells overexpressing PPARγ failed to form colonies in soft agar even in the absence of NSAIDs, a finding that suggests that these cells produce endogenous activators of PPARγ. Consistent with this finding is the observation of high basal levels of PPAR-RE promoter activity in NSCLC lines compared with normal lung epithelial cells (data not shown). Thus, the function of NSAIDs as PPARγ activators provides an appealing mechanism by which this class of drugs can inhibit the growth of diverse tumor cell types that fail to express COX or make prostaglandins.

Numerous studies have implicated a role for PPARγ in cancer, although the role of PPARγ in colon cancer is somewhat controversial. Loss-of-function mutations of PPARγ have been associated with development of sporadic human colon tumors (Sarraf et al., 1999), suggesting that PPARγ may function as a tumor suppressor gene. Consistent with this model, activation of PPARγ leads to inhibition of anchorage-independent growth of colon cancer cell lines (Brockman et al., 1998). By contrast, activators of PPARγ have been shown to promote development of colon tumors in $APC^{min}/+$ mice (Saez et al., 1998; Lefebvre et al., 1999), indicating a

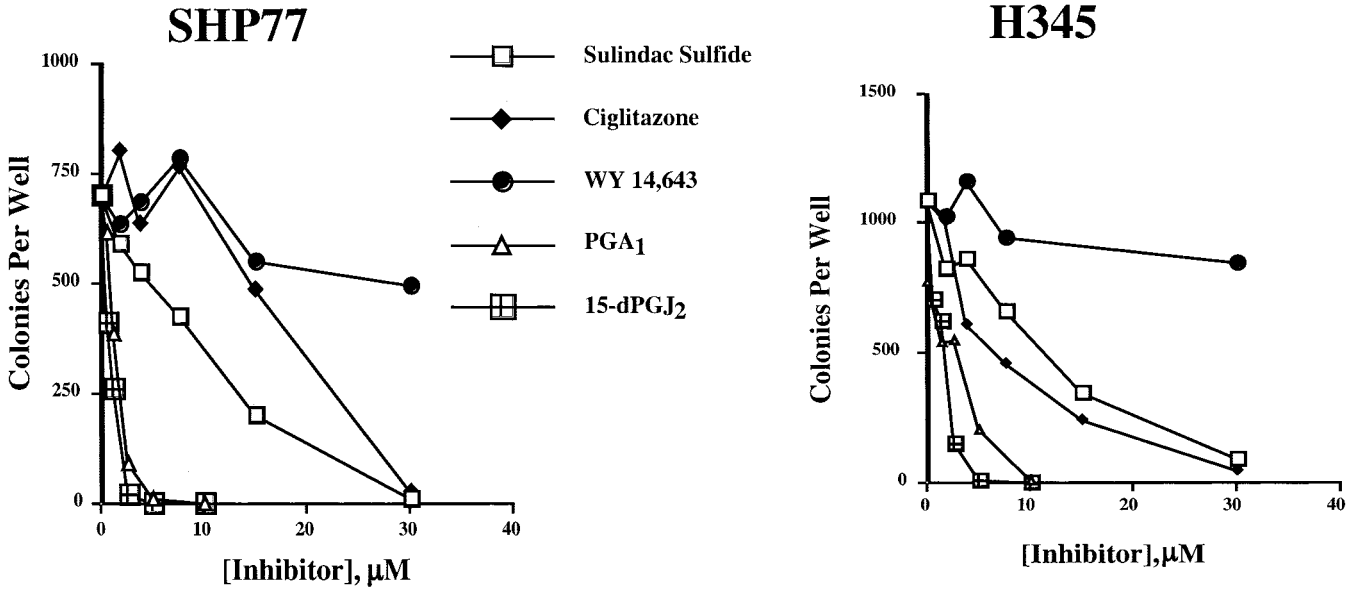

Fig. 6. Effects of NSAIDs and PPARγ activators on anchorage-independent growth of SCLC cells. Suspensions of SHP-77 cells (left) or H345 cells (right) were plated in soft agar in the presence of the indicated concentrations of various NSAIDs. Dishes were incubated for 3 to 4 weeks and colonies were counted under a microscope. Results represent the mean of three independent experiments.

tumor-promoting role for PPARγ. In NSCLC, ligands of PPARγ have been reported to induce differentiation and apoptosis (Chang and Szabo, 2000). Because PPARγ serves as a nuclear transcription factor, PPARγ activation in SCLC and NSCLC by NSAIDs would be predicted to increase transcription of genes whose products are either growth inhibitors, tumor suppressors, or proapoptotic. We have initiated studies to examine global changes in gene expression in response to overexpression and/or activation of PPARγ. Preliminary results have not identified cell cycle genes as being significantly changed by these maneuvers. However, increased expression of E-cadherin was observed both in response to drugs and in cells overexpressing PPARγ. Although the functional consequences of increased E-cadherin expression remain to be established, these data suggest that common genes activated through PPARγ may modulate the state of differentiation of these cells and thereby decrease tumorigenicity. Because sulindac sulfide, but not ciglitazone, inhibits eicosanoid production in NSCLC, we anticipate that changes in gene expression caused by activation of PPARγ and treatment with ciglitazone will not be identical, but overlapping families of genes should be regulated in common. It has recently been reported that PPAR-δ is also a target of NSAIDs (He et al., 1999). In those studies sulindac sulfide repressed expression of PPAR-δ-responsive promoters in colon cancer cells, suggesting that NSAID exposure may lead to both induction of pro-tumorigenic genes and suppression of antitumorigenic genes.

Finally, it should be noted that sulindac has been shown to act through additional pathways distinct from either COX inhibition or PPARγ activation. In oral squamous cell carcinomas, sulindac inhibited the expression and phosphorylation of Stat3 (Nikitakis et al., 2002). In contrast to our findings in lung cancer cells, sulindac inhibited activation of the extracellular signal-regulated kinase pathway in colon cancer cell lines (Rice et al., 2001). Activation of death receptor 5 and caspase-8 has also been implicated in sulindac sulfide-induced apoptosis in these cells (Huang et al., 2001). The existence of multiple targets for both NSAIDs and PPARγ activators suggests that care needs to be taken in attributing the action of these agents to specific molecular pathways. Alternative approaches combining molecular and pharmacological approaches will be required to delineate the contribution of individual pathways to inhibition of cancer cell growth. It is also likely that these pathways may have different roles in different cancer paradigms, necessitating a careful examination of each model.

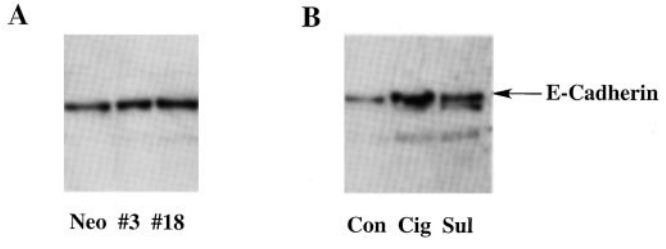

Fig. 7. Expression of E-cadherin in response to sulindac and PPARγ activation. A, cell extracts were prepared from H2122 cells stably transfected with empty vector (Neo) and two clones overexpressing PPARγ (3 and 18). Extracts were matched for protein and immunoblotted with an antibody specific for E-cadherin. B, untransfected H2122 cells were treated for 48 h with 40 μM ciglitazone (Cig), 50 μM sulindac sulfide (Sul), or vehicle (Con). Extracts were prepared and immunoblotted for E-cadherin. Both experiments are representative of three independent experiments.

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